Early Detection of Epithelial Ovarian Cancer via B7-H4 Quantification Within a Microfluidic System

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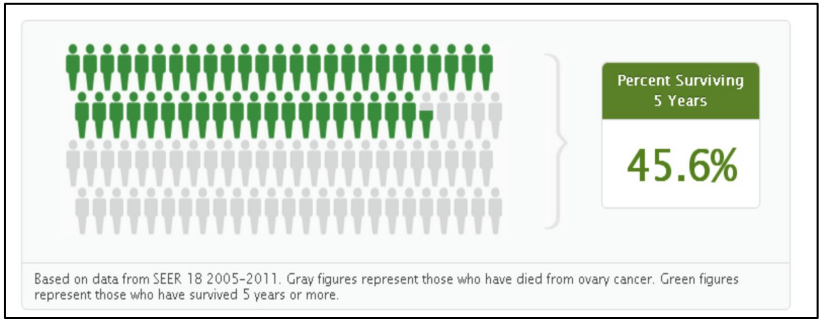
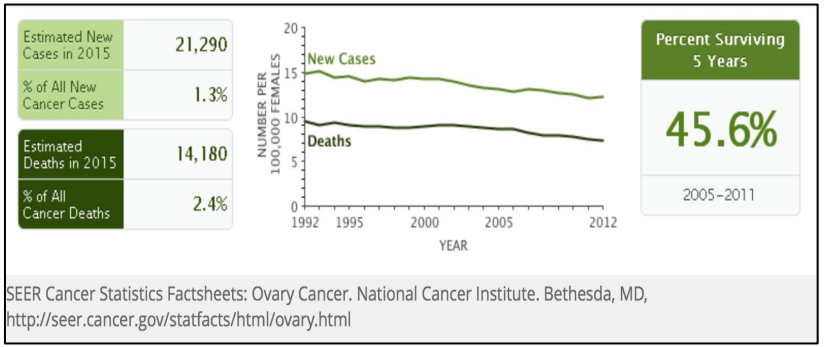
Quick Overview

- Ovarian cancer is the **fifth leading cause of death among women**, and is the deadliest of the gynecologic cancers
  - Only half of the women diagnosed with ovarian cancer survive
  - There is a 1 in 75 chance for any person of any ethnicity
  - Since the **"War on Cancer"** was declared, the mortality rates have only marginally decreased. [15]
    - While other cancer treatments have improved greatly, treatments and early-detection tests regarding ovarian cancer are lacking
- Treatment is reactive
  - Standard treatment is six rounds of chemotherapy
    - Only effective in 37% of women [15]
- Long-term treatment is necessary to battle ovarian cancer
  - Current early-detection tests are **costly** and too **insensitive**
  - No simple, cost-effective method of early diagnosis
  - No preventative mechanism only reactive
- To control the disease, there must be an early-detection test that doctors could use on their annual check-up of female patients





The graphs above from the Ovarian Cancer society illustrate that the mortality rates have only slightly decreased over the years and that less than half of the women diagnosed with ovarian cancer survived.

Past Biomarkers

(candidates for early-detection mechanism)

Cancer Antigen-125

Cancer Antigen-125 (CA-125) has been used as a biomarker for ovarian cancer

- a CA-125 level of 35 units to be a useful cutoff point, with 99% of healthy women having values less than 35 [9]

Problems

- CA-125 can be **absent even when a tumor is present**
- **Misdiagnosis**
  - Inflammatory conditions of abdomen also cause elevated levels of CA-125
- **Undetectable** at the early-stage
- **Cut-off marks deviate** greatly from patient to patient

Human Epididymis Protein 4

Human Epididymis Protein 4 (HE4) has been used as a biomarker for ovarian cancer

- More sensitive than CA-125
- Detectable in the bloodstream via an enzyme immunoassay with a cut-off level of 150 pmol/L [12]

Problems

- **Elevated in individuals with benign gynecologic conditions**, such as ovarian cysts
- Useful for monitoring not **screening**
- Higher levels in **postmenopausal** women
- Also **recorded for patients with renal failure** indistinguishable from ovarian cancer

Mesothelin

Mesothelin has been used as a potential biomarker for ovarian cancer

A 40 kDA secreted protein expressed in normal mesothelial cells and over-expressed in several human tumours

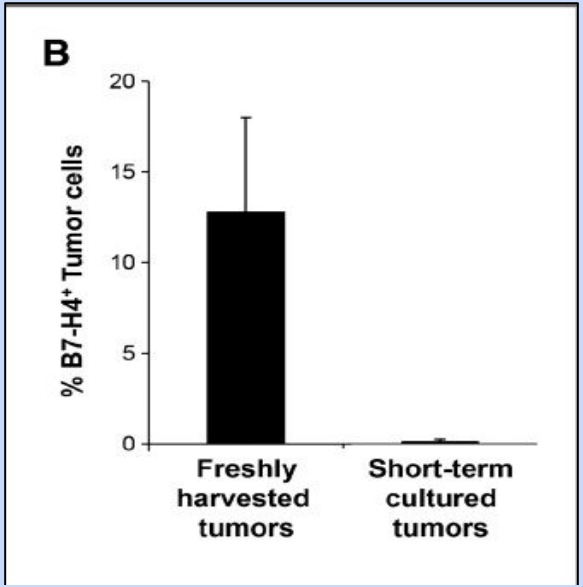
- Rejected simply because its presence was more **prominent in pancreatic cancer** than ovarian cancer [3]

B7-H4 - A New Biomarker

B7-H4: New, **More Specific** Biomarker

It has recently been discovered that B7-H4 is a protein mildly present in normal tissues but **highly concentrated in over 90% of cases** involving the malignant transformation of epithelial cells covering the ovary and abdominal cavity.

B7 family-homolog 4 (B7-H4) negatively regulates T-cell-mediated immune response when expressed on the surface of tumors by **down-regulating interleukin-2 production** and **arresting the cell cycles of both CD4+ and CD8+** T cells. [19] B7-H4 was readily expressed at the surface of freshly harvested young tumor cells, as shown in the image. *The Journal of Experimental Medicine* studied ovarian tumor macrophages and discovered that over seventy percent of freshly isolated tumor macrophages and tumor ascites CD14+ expressed cell surface B7-H4 protein while less than five percent of ovarian tumor cell lines expressed surface B7-H4. CD14 is a human gene encoding a protein component of the innate immune system. A tumor ascite is the buildup of fluid in the space surrounding the organs in the abdomen. Based on findings regarding cell surface expression of B7-H4, Dernada Dangaj and his team of data analysts hypothesize that **direct ovarian cancer cell eradication can be achieved by targeting B7-H4.**



Graph above from the paper, "Novel Recombinant Human B7-H4 Antibodies Overcome Tumoral Immune Escape to Potentiate T-Cell Antitumor Responses" This shows that B7-H4 is must more concentrated in freshly harvested tumors, necessary for early detection.

Decision

The final decision came by contemplating the research:

1. Ovarian cancer is the **fifth leading cause of death in women** globally
2. Current treatments are useful only after the cancer has proliferated
    a. Treatments are **not preventative**
3. The traditional biomarkers are either too **insensitive** or are **nonspecific** to ovarian cancer
4. Current tests are **expensive**, **infrequent**, and **take time**

Process

- Link a 50nm **iron oxide magnetic bead** to the monoclonal antibody via Fc Portion and a carbodiimide crosslinker.
- Link a **fluorescent nanoparticle** to the pair antibody via spontaneous amide to thiol reaction
- A **sandwich ELISA** test to convert an enzyme-linked anti-antibody into a detectable form and determine if the protein is present
- Create a model of a **microfluidic chip** that is compatible with the magnetic bead wherein a single blood sample is enough to test for B7-H4.

Goals of the Research

1. Create a **cost-efficient**, **quick**, and **user-friendly** test
2. A mechanism that uses sandwich ELISA to sensitively capture the protein and **quantify** its presence
3. Establish the **mechanism on a magnetic bead** instead of the traditional 96-well microplate
4. Manufacture a microfluidic chip in which the compacted detection mechanism can **analyze a single blood sample**

Research Overview

Fc Portion

Fc Portion achieves the **direct antibody conjugation** to a **nanoparticle** via carbohydrate moiety. Direct antibody conjugation involves two coupling reactions that converts chemical groups on the antibody and nanoparticle into a new formation that binds the two. Carbodiimides are used to begin the coupling reaction. Also called zero spacer arm cross-linkers, carbodiimides affect the conjugation of carboxyl groups to primary amines (lysine, N-termini), resulting in the formation of an amide bond that is shared between the groups. Carbodiimide cross-linkers react and activate the carboxylic acid groups on one molecule to form the active intermediate O-acylisourea. O-acylisourea reacts with the primary amine on the second molecule to form the amide. A common cross-linker is **1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC)** which activates carboxyl groups for spontaneous reaction with primary amines, enabling peptide immobilization and hapten-carrier protein conjugation [23].

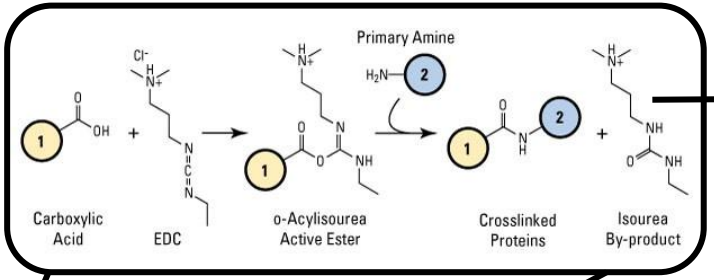
Sandwich Elisa

Enzyme-linked immunosorbent assay (ELISA): An ELISA test involves the **binding of a specific antibody to an unknown amount of antigen.** This detection antibody is then linked to an enzyme, and when a substrate is added, the enzyme-substrate complex triggers the enzyme to convert to some detectable signal, most commonly a colour change or chemiluminescence.

Sandwich ELISA: A sandwich ELISA test is favorable when **detecting a protein with two or more epitopes.** [18] It is suggested that either two different monoclonal antibodies be used or one monoclonal antibody and one polyclonal antibody, where the former would serve as the detection antibody. Additionally, sandwich ELISA is difficult to optimize, so for the purposes of the project, a tested **matched pair of antibodies** will be used to ensure that they are detecting different epitopes on the target protein and not interfering with each other's binding.

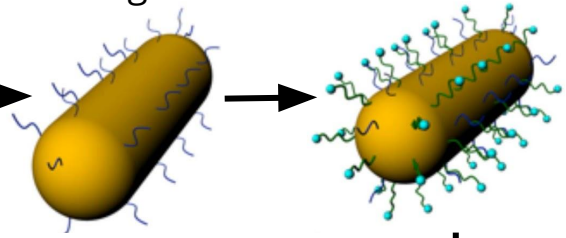
Process

Fc Portion

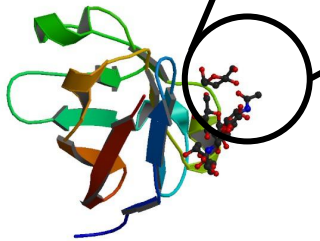


Iron-oxide magnetic bead

B7-H4 Antibody

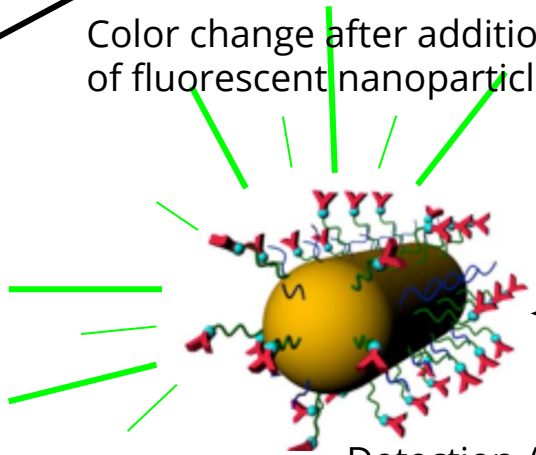


Capture Antibody



Capture Antibody

Color change after addition of fluorescent nanoparticle



Detection Antibody

Capture/Sandwich

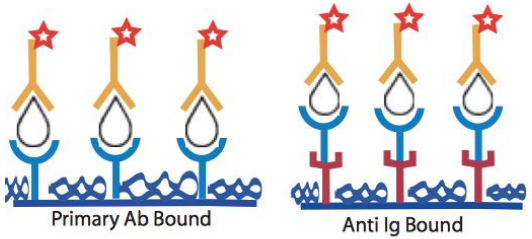
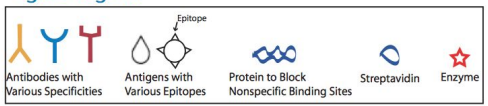
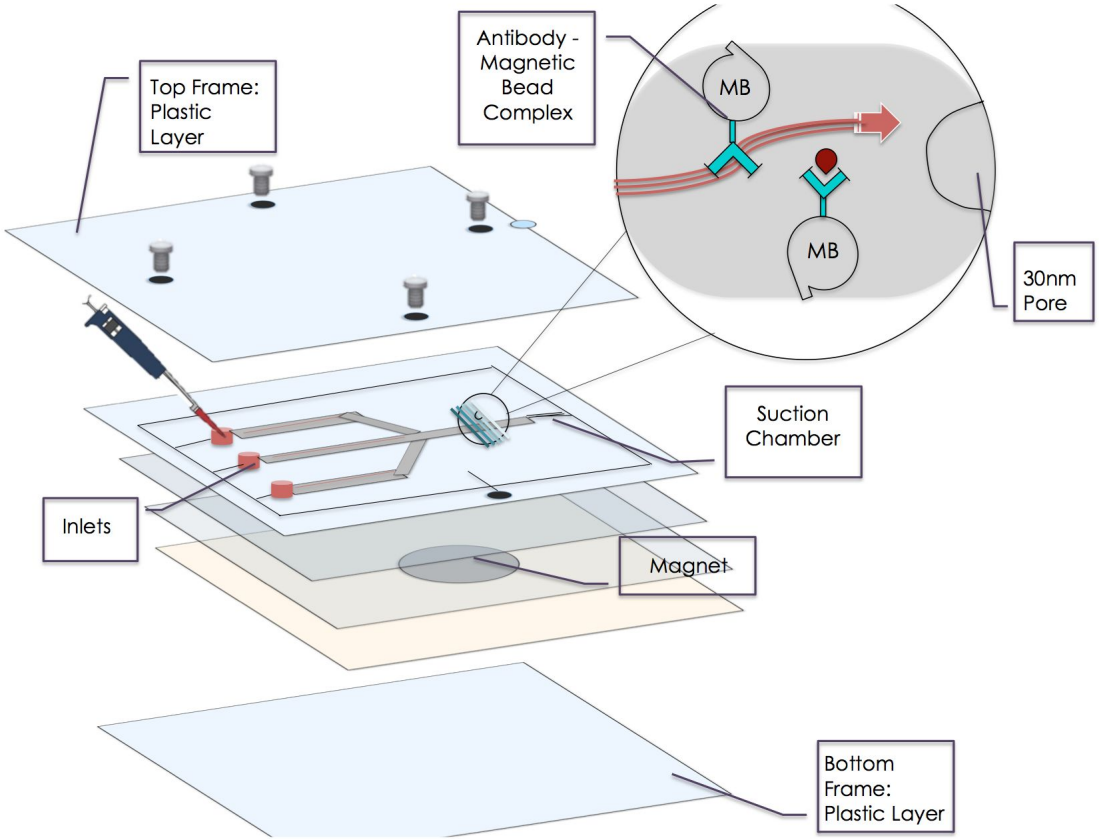


Figure Legend



Courtesy of intranet.tdmu.edu

Microfluidic Chip Model



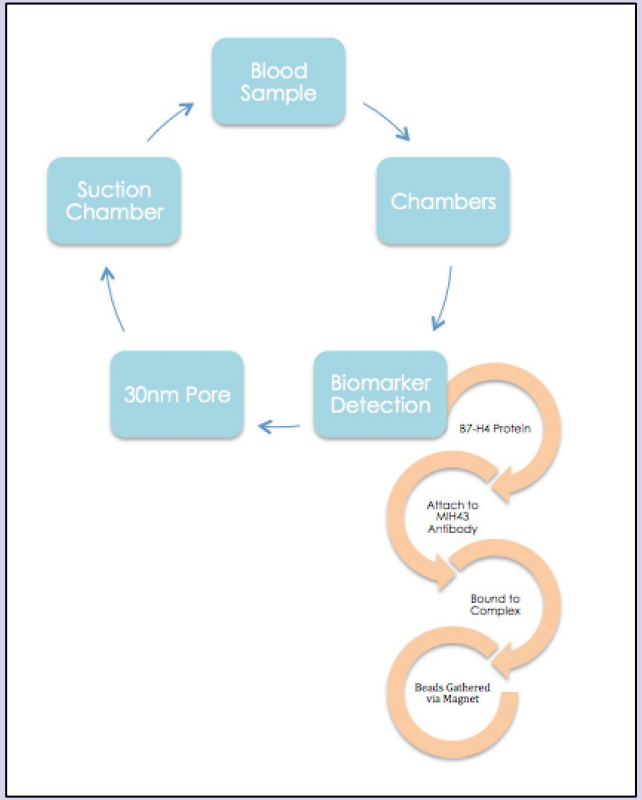
This diagram was self-made and displays a detailed description of how the microfluidic chip would work.

Key Points

- **Magnet** to attract the iron oxide magnetic beads that have the protein attached
- **3 inlets** to pipette in the blood
- **Plastic layer** to maintain low cost and increase flexibility of fluid movement
- **Suction chamber** to recycle the blood to increase the amount of successful bindings

Microfluidic Chip- How it works

1. Blood sample is pipetted into the inlets
2. Iron oxide magnetic beads are dispersed through the chambers
    a. Are linked to capture antibody via EDC
    b. 3 chambers to increase efficiency
3. Plastic sheets cover the magnet to prevent the beads from getting attracted right away
4. Blood passes through chambers
5. B7-H4, if present, captured by antibody, creating the particle-protein complex
6. Suction chambers recycle the blood
    a. 30nm to prevent the 50nm magnetic beads from getting sucked in
    b. Blood is recycled three times to increase the chances that the protein has to bind to the beads
7. Plastic coverings are removed to expose the magnet
8. Magnet attracts the protein-particle complexes
9. The complexes are isolated and added to fluorescent nanoparticle-detection antibody complex
10. Tested for B7-H4 presence through fluorescence intensity scan



Determining Optimal Antibody Concentration

Since purchasing antibodies are not cheap, the first step was to **determine the concentration of antibody that would allow for the maximum number of binding efficiency to the magnetic beads and the antigen**. For calculations, two different approaches were taken. First, the concentration of the microbeads, EDC and the antibody were calculated using their relative weights. Next, when keeping the volume of beads and volume of EDC the same, the relative volumes of antibody were calculated on a scale starting from 0.015μL of antibody to 15.301μL of antibody. A second method using the concentrations provided on the product website confirmed that the initial calculations were accurate.

Preparing the Samples

A total of 12 tubes were prepared, one with just the buffer and the beads to serve as a negative control. In each tube, the same amount of beads and EDC was added. The amount of antigen was added by diluting the concentration of antigen by half in each subsequent tube. The EDC was first prepared in an MES buffer and then washed with PBS. The reaction was left for 15 minutes and then rinsed with a magnet. The rinsing process was repeated 3 times.

The entire procedure was repeated 3 times to ensure that the results were reliable.

number of vial	mix ratio of Ab/Mb	volume of Ab (uL)	volume of Mb (uL)	EDC/Mb	vol of EDC (uL)
1	1	0.015	10.0	2.1E+07	50.00
2	2	0.030	10.0	2.1E+07	50.00
3	4	0.060	10.0	2.1E+07	50.00
4	8	0.120	10.0	2.1E+07	50.00
5	16	0.239	10.0	2.1E+07	50.00
6	32	0.478	10.0	2.1E+07	50.00
7	64	0.956	10.0	2.1E+07	50.00
8	128	1.913	10.0	2.1E+07	50.00
9	256	3.825	10.0	2.1E+07	50.00
10	512	7.651	10.0	2.1E+07	50.00
11	1024	15.301	10.0	2.1E+07	50.00

Table shows the contents of each test tube with the volume of antibody varying while the amount of beads and EDC remained the same.

BCA Protein Assay

A bicinchoninic acid (BCA) protein assay was performed to provide a general idea of the **total bound protein concentration in a sample containing the iron oxide magnetic beads, capture antibody, and B7-H4.** This assay gives less sensitive results, however, the vibrant purple-colored reaction makes it easy to determine the holistic success of the protocol.

The Process

The assay was done five separate times, each time accounting for a confounding variable. Three separate amounts of a 30μL sample were included : 10.00μL, 2.00μL, and 0.500μL . The negative control was given by the iron oxide bead in PBS 1X buffer. A blank with just PBS was also included. The standards were in-stock. The working reagent was made with 200.00μL of the Pierce BCA Protein Assay Reagent A and 10.00μL of the Pierce BCA Protein Assay Reagent B to make a 20:1 ratio. The standards and samples were pipetted in a span of 10 minutes for the rate of the color development at room temperature to remain insignificant. The microplate was left to incubate for 30 minutes at 37°C.

Changes In Each Trial

Trial 1: Three concentrations of sample were used - 10.00 μL, 2.00μL, and 5.00

Trial 2: Resuspension of the particles was accounted for through vigorous shaking prior to aliquoting in order to place roughly similar concentrations of the particle-protein complex with respect to the sample in each well

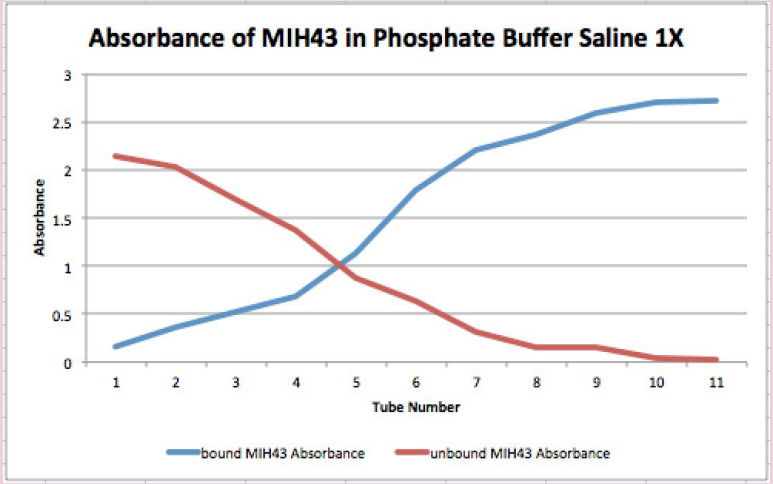
Trial 3: Three wells containing 2.00μL of the sample were tested along with 10.00μL and 0.500μL of sample

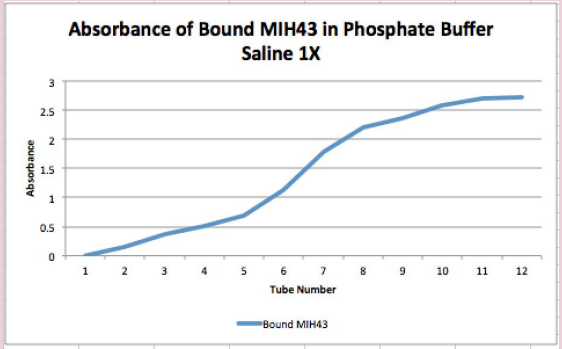
Trial 4: Replacing the forward pipetting technique with reverse pipette

Trial 5: Redid the entire procedure taking into account all of the changes.

Results of Antibody Test

The graph on the right depicts the absorbance of capture antibody when varying volumes are included in the solution. Absorbance was determined using the Eppendorf Bio Photometer Instrument. The absorbance of the beads alone was subtracted from each absorbance of the bound antibody. The graph shows that the **optimal concentration of capture antibody that maximizes the number of successful coupling reactions occurred in Tube 10, which had 40uL of capture antibody and 100uL of PBS**. In addition, the data demonstrates that with each dilution beginning with Tube 11 and ending with Tube 1, the absorbance decreased by roughly half of the previous number. The S shape is also an indicator of positive results because it signifies the maximum absorbance of the capture antibody regardless of a concentration above 10uL.





The graph on the left shows the absorbance of just the bound capture antibody. The bound absorbance was determined by isolating the beads with a magnet and then pipetting the solution into a cuvette, which was then placed into the photometer. The **unbound absorbance was given by taking the supernatant of each tube and calculating its relative absorbance.** The graph illustrates that the amount of unbound antibody gradually decreased from Tube 11 to Tube 1, which implies that an increasing amount of antibody was binding to the iron oxide magnetic beads as expected.

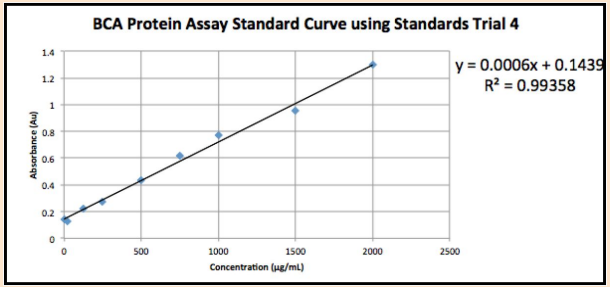
Results of BCA Protein Assay

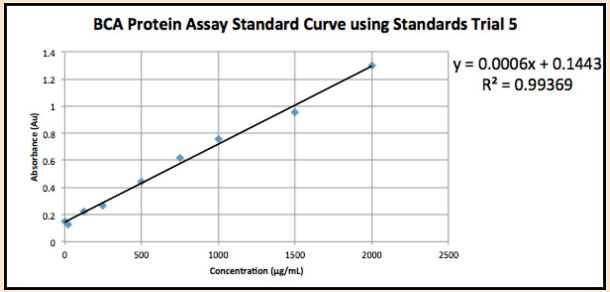
Standard Concentrations (μg/mL)	Absorbance of Standard Concentrations (Au)	Samples	Absorbance of Samples (Au)	Calculated Concentration of Samples (μg/mL)	Accounting for Dilution Factor (ug/mL)
2000.00	1.2988	Blank - with PBS buffer	0.1675	39.33333	--
1500.00	0.954	Negative Control - Iron Oxide Particle in PBS 1X buffer	0.1567	21.33333	--
1000.00	0.7692	10.00μL of sample	1.052	1513.5	1513.5
750.00	0.6201	2.00μL of sample	0.3243	300.6667	1503.333
500.00	0.4327	2.00μL of sample	0.3256	302.8333	1514.167
250.00	0.273	2.00 μL of sample	0.3269	305	1525
125.00	0.2193	0.500μL of sample	0.1887	74.66667	1493.333
25.00	0.1278	Empty	--	--	--
0.00	0.1453				

Assay on Particle-Protein Complex Trial 4

Standard Concentrations (μg/mL)	Absorbance of Standard Concentrations (Au)	Samples	Absorbance of Samples (Au)	Calculated Concentration of Samples (μg/mL)	Accounting for Dilution Factor (μg/mL)
2000.00	1.2997	Blank - with PBS buffer	0.1556	18.83333	--
1500.00	0.952	Negative Control - Iron Oxide Particle in PBS 1X buffer	0.1578	22.5	--
1000.00	0.7602	10.00μL of sample	1.053	1514.5	1514.5
750.00	0.6213	2.00μL of sample	0.325	301.1667	1505.833
500.00	0.4431	2.00μL of sample	0.3258	302.5	1512.833
250.00	0.269	2.00μL of sample	0.3252	301.5	1507.5
125.00	0.2201	0.500μL of sample	0.1894	75.16667	1503.333
25.00	0.1273	Empty	--	--	--
0.00	0.1456				

Assay on Particle-Protein Complex Trial 5





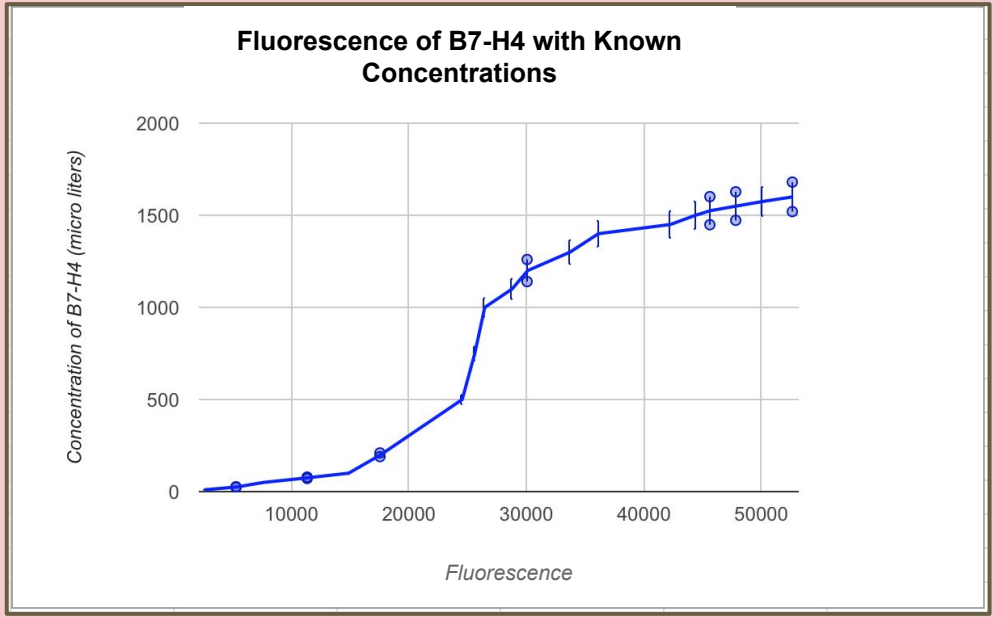
Results from the fourth trial were very accurate and consistent with the expected numbers. The **concentrations of B7-H4 were all very close to 1510μg/mL** and the 2.00μL sample produced a concentration in between the 10.00μL sample and the 0.500μL sample as expected. Additionally, the absorbance of the three 2.00μL samples were very close,

The reverse pipetting technique was successful. **A fifth trial that mimicked the exact protocol of the previous trial yielded positive results as well, indicating that our results were not caused purely by chance.** A significance test of the concentrations from trials 4 and 5 showed no convincing evidence **(p = 0.896)** that the true concentration of B7-H4 in 10.00μL of sample deviated significantly from 1510μg/mL. Additionally, at C = 95 the obtained concentration of 1510μg/mL falls within the calculated interval, suggesting that the true mean is extremely close to the experimental value

Elisa Test Results

The graph on the right shows the results from the sandwich ELISA procedure. The distinct S-shape indicates a positive test result. The graph begins to level off at around 1450μL which is consistent with the results from the BCA assay which centered around 1510μL. Starting at 1400μL of B7-H4, the intervals between the known concentrations of B7-H4 became smaller because we hypothesized from the BCA protein assay that the curve should level off. Additionally, low concentrations of B7-H4 corresponded with a low fluorescence while high concentrations resulted in a high fluorescence. This confirms the fact that B7-H4 can be used as a biomarker for ovarian cancer and that the sandwich Elisa test is a viable option to test for the presence of the protein.

This procedure was repeated three times, each time resulting in a concentration around 1450μL. Since many of the potential sources of error were solved when completing the BCA protein assay, we found the results of each trial more consistent with the ELISA test. The specificity of this assay solidifies the mechanism and proves that the particle-protein complex was successfully linked.



Graph above shows the results from the Elisa assay. The error bars represent a 5% error for each concentration. 19 concentrations were tested along with a blank to serve as the control.

Conclusion

For the past year, we have strived hard to meet our research goals. After experiencing failures and success alike, here are the conclusions

Antibody Concentration

- Determined that **10.00μL** was the optimal concentration **of capture antibody** to add to the magnetic bead solution.

Sandwich ELISA

- Results were consistent with literature reviews citing an **increased fluorescence for higher concentrations of B7-H4**
- Allows for **simple classification of malignancy** or no malignancy
- In cases of continuous surveillance, the **mechanism lends itself to be run many times** with consistency

B7-H4 Detection

- Mechanism **specific to B7-H4** and **independent of the magnetic beads**, which ensures that the noninvasive mechanism serves it's early-detection purpose
- **Magnetic beads are compatible with the microfluidic system**, greatly conserving the use of expensive chemicals and pre-made kits

Microfluidic Chip

- **Quick** -- eliminates the length timeframe of current lab-based ELISA tests
- **Cost-effective** -- requires a fraction of the cost of current early-detection tests while allowing for an unlimited number of samples to be tested
- **Simple** -- only a single, noninvasive blood sample is necessary

Final Remarks

With further testing, women will now have the opportunity to be checked for ovarian cancer during their annual doctor's visit.

The test can be done with just a single blood sample that is pipetted into the chip.

The speed with which this test can be conducted and the low cost allows for women to greatly reduce their risk of developing ovarian cancer and gain immediate health feedback.

A few more refinements will allow for a preventative treatment to exist for ovarian cancer.

Future Work

Future work largely includes creating the microfluidic chip based on the model that was developed. The UCSD lab that we currently work at has a 3D printer that will allow us to create the parts that we need. Additionally, a team specializing in microfluidic chip design has agreed to help us make our model a reality, and we are in contact with Ignyta, a biotech company based in San Diego.

In addition, we hope to run patient samples through the chip and compare them with the ELISA test results we obtained. The results will serve as the standard curve which we can then use to compare against the patient samples with unknown concentrations of the protein. Before we run patient samples however, we are planning on using mouse plasma, which is similar to human plasma but much cheaper. Plasma contains proteins found in the blood, and we will spike it with varying known concentrations of B7-H4 to determine the success of isolating and detecting B7-H4 amongst other background noise. This will give us alternate results to those given by our engineered samples.

Furthermore, we are interested in seeing if the different stages of ovarian cancer can be determined by the relative concentration of B7-H4 that is calculated with the chip. We hypothesize that a higher concentration of B7-H4 will correspond to a stage IV tumor. However, we are curious if the amount by which the concentration of B7-H4 increases is significant enough to detect.

Moreover, we are in communication with the Ovarian Cancer Research Fund and the Rivkin Center and are looking to share our research with the ovarian cancer community. Being part of the community ourselves, our main goal is to improve the lives of the patients and their loved ones. With this research, we aim to have a better understanding of this specific cancer so that it can eventually be stopped.

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